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14. ABSTRACT Some biological sensors undergo structural changes, such as the opening of a large pore, upon matrix deformation. Other biological molecules have the ability to bind heavy metals, such as nickel, cadmium, copper, etc. We have been inspired by these findings to proposed a fusion of these biological attributes into a single nanosensor. The long-range goal of this project is identify conditions in which we can utilize a biological mechano-sensor, MscL, to engineer a nano-scale mechanically-activated solid-state relay device. To do this, it would be preferable to design a metal, or nanoparticle, binding site within the pore of this biological sensor that allows electrical conduction only upon mechanical-stress of the matrix in which it is embedded. This would allow stability under conditions of little moisture. Using funding from the BioInspired Concepts (BIC) program of the AFOSR, we have utilized biochemical and electrophysiological assays to identify regions of the sensor thar are exposed (SEE ATTACHED.)					
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A) Overview:

Some biological sensors undergo structural changes, such as the opening of a large pore, upon matrix deformation. Other biological molecules have the ability to bind heavy metals, such as nickel, cadmium, copper, *etc.* We have been inspired by these findings to propose a fusion of these biological attributes into a single nanosensor. The long-range goal of this project is identify conditions in which we can utilize a biological mechano-sensor, MscL, to engineer a nano-scale mechanically-activated solid-state relay device. To do this, it would be preferable to design a metal, or nanoparticle, binding site within the pore of this biological sensor that allows electrical conduction only upon mechanical-stress of the matrix in which it is embedded. This would allow stability under conditions of little moisture.

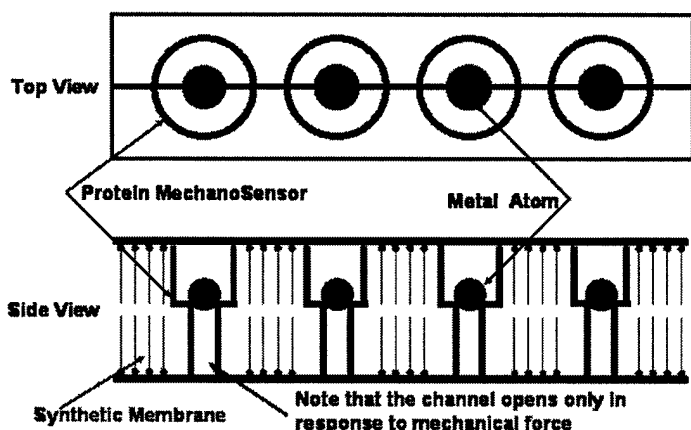


Figure 1) Potential design of nano-scale mechanically-activated solid-state relay device. (courtesy of Dr. Morley Stone, Biotechnology Group Leader, AFRL/MLPJ)

Using funding from the BioInspired Concepts (BIC) program of the AFOSR, we have utilized biochemical and electrophysiological assays to identify regions of the sensor that are exposed in the pore lumen. We have mutated these candidate sites to cysteine and histidine residues, amino acids that are known to bind heavy metals. Electrophysiological studies have now confirmed that some of these substituted residues within the sensor do indeed bind metals; however, many of these interactions deleteriously affect the probability or energetics of sensor activation. Hence, we needed to define those regions exposed in the open pore of the stimulated channel. To

accomplish this, we have designed and used microbial screens to determine candidates for residues that are exposed only upon sensor activation.

B) Background

B1) MscL as a mechanosensor

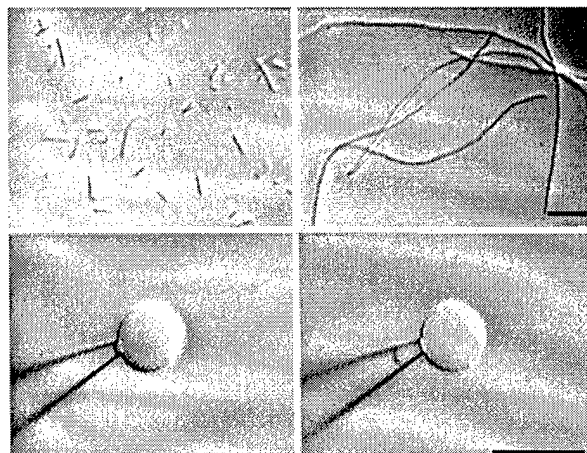


Figure 2. Patching bacterial membranes. Utilizing drug and enzymatic treatments, bacteria are grown into long filamentous cells as shown in the top right panel, then collapsed into spheres as shown in the bottom panels (Blount et al., 1999), a small amount of membrane is taken into a glass electrode, a voltage potential is established across the membrane, the membrane is stimulated, and channel activities, as presented in Fig. 2, are recorded.

Much is known about bacterial sensors (Blount and Moe, 1999; Blount et al., 1999; Blount, 2003). The best-defined sensor, MscL, serves as an “emergency release valve” to protect the cell from rapid decreases in osmotic environment. To prevent lysis, the MscL sensor opens a large pore that allows the cell to rapidly jettison compatible solutes from the cytoplasm to the external medium. MscL activity was first identified by the patch-clamp technique (**Fig. 2**). Here, the small bacterial cells are inhibited from dividing to generate long filamentous cells that are then collapsed into large spheres; a small amount of bacterial membrane is taken up in a glass electrode; a voltage potential is placed across the membrane; the sensor is stimulated and channel activities are measured by recording ionic current, as presented in **Fig. 3**. As shown in **Fig. 3**, we normally use pressure, which leads to membrane tension, to stimulate this sensor. Note that the “gating” of the pore of this molecule is in contrast to α -haemolysin and other toxin channels,

which are always in the open state. It is this gating to a specific stimulus that makes MscL uniquely appropriate for the generation of a nanosensor.

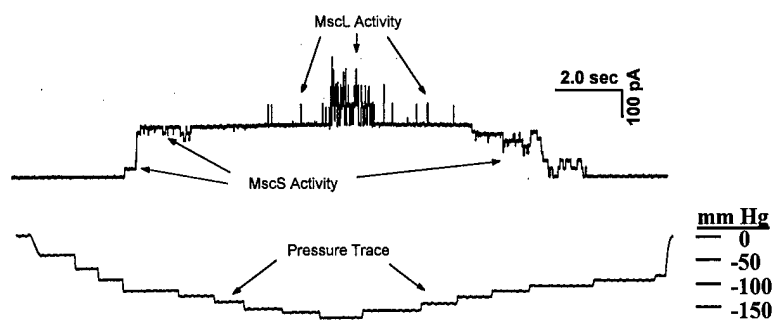


Figure 3. A typical current trace from a patch derived from an *E. coli* spheroplast. MscS and MscL activities (top) and the pressure trace (bottom) are shown. The pressure is generated by suction in the patch electrode; pressure relative to atmospheric is shown in mm Hg. (from Blount, *et al.*, 1996)

We have successfully reconstituted the MscL activity into an artificial system. In these studies, we solubilized *E. coli* membrane proteins with a mild detergent, and reconstituted them into purified or synthesized lipids (Moe and Blount, 2002). Note that during the reconstitution of the sensor into lipids, we used a dehydration and rehydration procedure. These studies demonstrated that the MscL sensor activity can survive all of these insults and could still be readily assayed by patch-clamping the resulting liposomes. In fact, it was using this observation, and subsequent biochemical enrichment of the activity, that allowed us to identify the MscL protein and its corresponding gene (Sukharev *et al.*, 1994). In sum, we have demonstrated that the sensor-protein can be molecularly tagged, purified, and reconstituted into artificial membranes, yet remain functional (Blount *et al.*, 1996b; Moe and Blount, 2002).

Since it was first identified, much has been learned about the physiology, activity and structure of the MscL sensor. The MscL sensor opens a very large pore in response to stimulation. The pore size is estimated to be 30 to 40 Å in diameter (or a volume of approximately 175 cubic microns, assuming a cylinder); this gives MscL the largest known biological gated pore. We have generated many mutants of this sensor that have altered properties, including kinetics of activity, threshold of response and sensitivity. Finally, it remains one of a handful of gated biological pores to have the structure defined to near atomic resolution

(3.5 Å) by X-ray crystallography (Chang et al., 1998). The protein backbone for the structural model for the crystallized MscL molecule is shown in Fig. 4.

The crystallized MscL is actually an orthologue of the *E. coli* sensor derived from the organism *Mycobacterium tuberculosis*. Because this orthologue is difficult to activate (Moe et al., 2000), and all of the mutants and physiological studies exist only for the *E. coli* sensor, we have been working with the latter and transposing the structural features of this orthologue onto the *E. coli* sensor to derive our models.

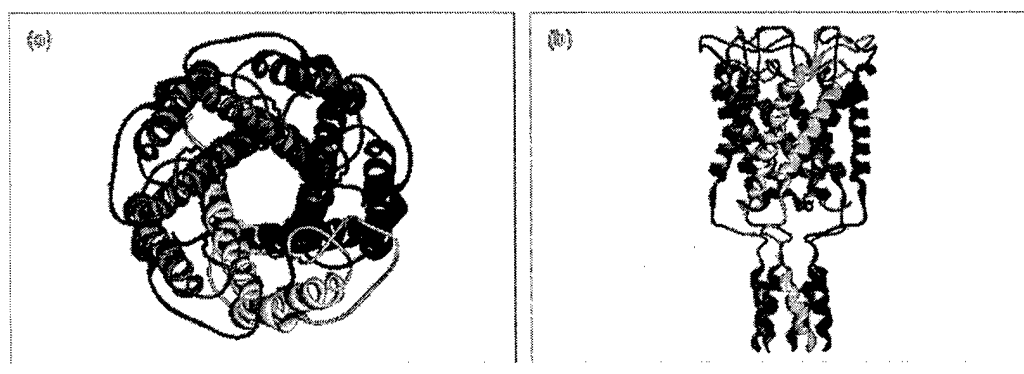


Figure 4. The structure of MscL as defined by X-ray crystallography. Panel (a) to the left shows a view looking down on the molecule. Panel (b) to the right shows a side view of the molecule; the top bundle of helices lies in the membrane while the lower helices are in the cell cytoplasm. Note that the entire complex is made of five identical subunits. (From Moe and Blount, 1999)

In summary, the MscL channel has many of the properties desired for utilization in a nanosensor:

- 1) Sensitivity to mechanical forces
- 2) Large conformational changes upon activation
- 3) Existence of a number of mutated sensors with various properties and activities
- 4) The potential to utilize microbial genetics to facilitate the directed engineering of sensors with specific and desirable properties

- 5) A resolved structure allowing for the directed manipulation of segments or domains of the sensor
- 6) Resilience to biochemical manipulations; the ability to be purified, dehydrated, reconstituted into artificial systems and yet remain functional

B2) Previous models for the structure of the activated sensor

Although the crystal structure has advanced the field tremendously, several questions remain. One is whether the structure resolved corresponds to the fully inactive (closed) state of the channel. In the structure V21, V23 in *E. coli*, was determined to be the constriction point on the periplasmic side of the pore. However, using a cysteine scanning approach, we found that a G26C mutant in the *E. coli* MscL appeared to more efficiently form disulfide bridges when in its native membrane environment than V23C, as determined by the percentage of channel activities revealed by the reducing reagent DTT. The only other residue, in a full scan of both TM domains, to display the property of a 'locked-closed' channel in the absence of DTT was R13C. Another issue has been what structural changes occur during the transition of closed to open states. Modeling of possible open states led to the proposal of an iris-like opening of the channel in which the TMs tilt and the first transmembrane domain lines the open pore (Sukharev et al., 2001). This aspect of the model has been supported by **electron paramagnetic resonance (EPR)** spectroscopy. Disulfide trapping experiments have suggested that the two TMs can be attached and maintain an interaction in all states of the channel, suggesting that TM1 undergoes a slight counterclockwise rotation (as viewed from the periplasm) during gating. However, the EPR studies suggested that TM1 may undergo a full 110° rotation in the *opposite*, clockwise

direction during this transition. We have been testing these hypotheses by independent means, and thus far our data support the EPR predictions, bringing into question the opposing theory.

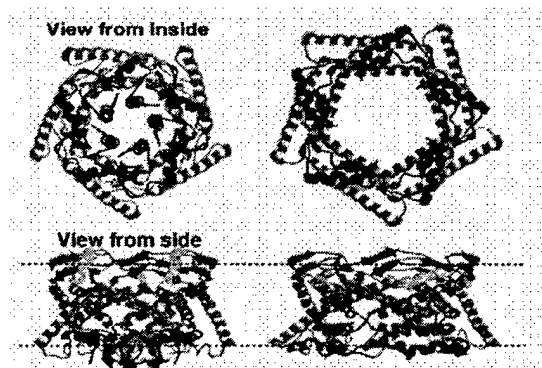


Fig. 5) A proposed model for the closed-expanded (CE) (left) and fully open (right) structure of MscL. It is generally agreed that the TM domains tilt with respect to the membrane and that TM1 forms the majority of the pore. However, the stability of the CE state and the rotation of TM1 during gating remain in dispute. (From (Sukharev et al., 2001)).

An additional proposal that has been made is that the TM1 helices separate and form a large vestibule prior to channel opening; this state is referred to as the **Closed-Expanded (CE)** state. By this model, the extreme N-terminal region of the protein (S1), which was not resolved in the crystal structure, serves as the ultimate ion permeation barrier: the removal of each of the S1 domains from the cluster leads to the observed substates. However, several lines of evidence now suggest that the CE state of the molecule cannot, as predicted, be a stable state of the molecule. First, mutations within the first transmembrane domain, but not the S1 domain, affect the kinetics of the channel. If the CE state were truly stable, it is difficult to understand how TM1 mutants, which should only change the stability of CE state, could influence channel open dwell times to shorter or longer, depending upon the TM1 mutation. Second, of the numerous genetic experiments performed and mutants isolated, none that confer **gain-of-function (GOF)** phenotypes (channels more sensitive to membrane tension), or **loss-of-function (LOF)** phenotypes (channels requiring more membrane tension or that are non-functional) were found in the S1 domain, and only 1 of the 28 mutants isolated as intragenic suppressors of GOF mutants was found in this domain. On the other hand, studies have shown that the S1 domain is necessary to form functional channels; no channel activities are observed if this domain is deleted (Blount et al., 1996a; Blount et al., 1996c). Hence, the precise function of the N-terminal

S1 domain is still unclear. One of the current models (**not** derived from a crystal structure) is shown in **Fig. 5**.

C) Progress

In the past three years, we have made substantial strides in generating models for the activated state of the MscL sensor. As described below, several projects, some independently funded, have fed into the project: to design metal binding sites within the channel (as described by our recent publication on the topic (Iscla et al., 2004)). As described below, information derived from a cysteine library has given us clues and reagents to measure metal binding to what we think is inside the pore. In addition, it has allowed us to use a novel approach, coined an *in vivo* SCAM, to determine residues exposed in the activated sensor. The following are our publications citing this AFOSR funding (excluding meeting abstracts):

1. Okada, K., Moe, P. C., and Blount, P. (2002). Functional design of bacterial mechanosensitive channels. Comparisons and contrasts illuminated by random mutagenesis. **J. Biol. Chem.** 277, 27682-27688.
2. Li, Y., Moe, P. C., Chandrasekaran, S., Booth, I. R., and Blount, P. (2002). Ionic regulation of MscK, a mechanosensitive channel from *Escherichia coli*. **EMBO J** 21, 5323-5330.
3. Kumánovics, A., Levin, G., and Blount, P. (2002). Family ties of gated pores: evolution of the sensor module. **FASEB J** 16, 1623-1629.
4. Poolman, B., Blount, P., Folgering, J. H., Friesen, R. H., Moe, P. C., and van der Heide, T. (2002). How do membrane proteins sense water stress? **Mol. Microbiol.** 44, 889-902 (invited review).
5. Moe, P. C., and Blount, P. (2002). A novel approach for probing protein-lipid interactions of MscL, a membrane-tension-gated channel. In Biophysical chemistry; membranes and proteins, R. H. Templer, and R. Leatherbarrow, eds. (Royal Society of Chemistry (UK)), pp. 199-207 (invited symposium chapter).
6. Blount, P. (2003). Molecular mechanisms of mechanosensation: big lessons from small cells. **Neuron** 37, 731-734 (invited review).
7. Levin, G., and Blount, P. (2004). Cysteine scanning of MscL transmembrane domains reveals residues critical for mechanosensitive channel gating. **Biophys J** 86, 2862-2870.
8. Li, Y., Wray, R., and Blount, P. (2004). Intragenic suppression of gain-of-function mutations in the *Escherichia coli* mechanosensitive channel, MscL. **Mol Microbiol** 53, 485-495.
9. Bartlett, J. L., Levin, G., and Blount, P. (2004). An *in vivo* assay identifies changes in residue accessibility on mechanosensitive channel gating. **Proc Natl Acad Sci U S A** 101, 10161-10165.

10. Iscla, I., Levin, G., Wray, R., Reynolds, R., and Blount, P. (2004). Defining the physical gate of a mechanosensitive channel, MscL, by engineering metal-binding sites. **Biophys. J.** 87(5), 3172-3180.
11. Kung, C., and Blount, P. (2004). Channels in microbes: so many holes to fill. **Mol Microbiol** 53, 373-380 (invited review).
12. Folgering, J. H. A., Moe, P. C., Schuurman-Wolters, G. K., Blount, P., and Poolman, B. (2004). *Lactococcus lactis* uses MscL as its principal Mechanosensitive channel. **J Biol Chem Epub ahead of print**, M411732200.
13. Blount, P., Iscla, I., Li, Y., and Moe, P. C. (in press). The bacterial mechanosensitive channel MscS and its extended family. In *Bacterial channels and their eukaryotic homologues*, A. Kubalski, and B. Martinac, eds. (Washington, D.C., ASM Press) (invited review).
14. Edwards, M. D., Li, Y., Kim, S., Miller, S., Bartlett, W., Iscla, I., Blount, P., Bowie, J. U., and Booth, I. R. (in press). Gating the MscS mechanosensitive channel: pivotal role of the glycine-rich sequence. **Nature Struct. Mol. Biol.**

C1) Generation and use of the cysteine library

We have generated single cysteine substitutions along the entire two transmembrane domains: TM1 and TM2. As described below, functional assays verified the importance of residues in the putative primary gate in TM1, corroborate other residues previously noted as critical for normal function and identified new ones. In addition, evaluation of disulfide bridging in native membranes suggested alterations of existing structural models for the “fully closed” state of the channel. Preliminary experiments utilizing sulfhydryl reagents now identify residues that may be exposed in the open pore upon gating. This information has been used to design metal binding sites within the pore of the sensor.

C1i) Identification of gain-of-function (GOF) and loss-of-function (LOF) mutants and their implications in functional regions gating dynamics

Cysteine scanning has been utilized previously to determine functional regions within transmembrane domains (Lee et al., 1995). We have used this approach to determine residues in TM1 and TM2 that play important roles in the gating of MscL. Because wild type *E. coli* MscL contains no cysteines, each substituted cysteine is unique within the subunit. Cysteine was chosen for scanning such domains because its hydrophobicity lies near the middle range for

transmembrane residues and its relatively small size means that perturbation of packing interfaces would likely be subtle. In addition, it is found in every protein secondary structure and is therefore unlikely to distort global structure. Hence, when a cysteine substitution does lead to phenotypic changes, it suggests that some feature of the substituted residue (e.g. size, hydrophilicity, aromatic ring or charge) is important for normal channel function. Utilizing this approach, combined with whole-cell physiological and electrophysiological methods, we confirmed the importance of TM1, and implicated the participation of TM2 in MscL gating. Many of the mutations that effected the most severe phenotypes were in the middle of TM1, confirming the importance of the proposed primary gate (V21 to A27), and at the cytoplasmic and periplasmic ends of TM2, suggesting that protein-lipid interactions in this portion of the domain may be important.

C1ii) Disulfide bridges that form efficiently enough to disrupt channel activity in patch clamp.

All channels that led to a severe phenotype were assayed by patch clamp for channel activity. One significant finding was that hydrophilic changes in TM1 that conferred a GOF phenotype also showed activities with shorter dwell times. These data are again consistent with the hypothesis that TM1 (not the N-term, S1 domain) is the primary gate for ion permeation, making the CE state unlikely. Another important finding was that two mutants that effected a GOF phenotype did not appear to encode channel activity; however, upon treatment with DTT, activities consistent with a GOF phenotype were revealed **Fig 6**). This apparent paradox: channels are locked-closed in patch clamp but mis-gate *in vivo*, could be explained by the already reduced cellular environment (Carmel-Harel and Storz, 2000; Ritz and Beckwith, 2001).

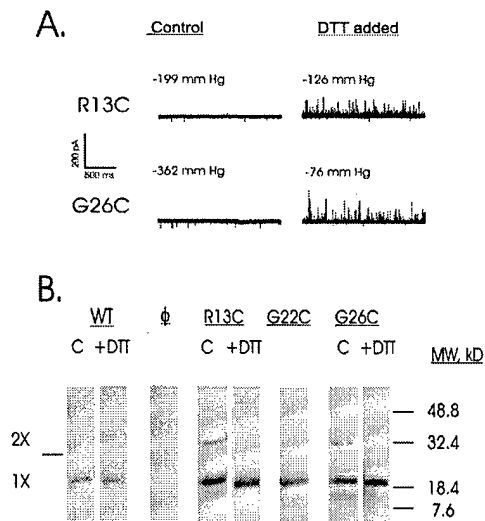


Fig. 6) Substitutions R13C and G26C spontaneously form disulfide bridges. **A.** Typical traces of R13C (top) and G256C (bottom) are shown before (left) and after (right) addition of 3 mM DTT. **B.** Western blot analysis of MscL mutants and controls. Where indicated, the samples were treated with DTT. The pB10 expression plasmid, not containing *mscL*, is shown as a control (labeled Φ). These data led to the hypothesis that G26, not V23, forms the constriction point of the closed channel. Hence, the crystal structure reflects a “nearly-closed” rather than “fully-closed” state of the channel. We are now acquiring additional evidence for this hypothesis. (From (Levin and Blount, 2004)).

One of the mutations that had apparently formed disulfide bridges in patch clamp was at R13, a region of the protein that is still poorly understood. We hypothesize that these residues must approach each other during gating: the charges normally effect an electrostatic repulsion and thus an energetic barrier to opening; their removal of leads to the GOF phenotype. The other disulfide-bridging residue was near the constriction point of the channel, G26. However, if the crystal structure is of the closed state, then one would predict that V23, not G26 would be in proximity and face each other. Given this finding, in combination with the previous findings that there was still a 4Å hole within the crystallized MscL complex, and that EPR studies suggested that TM1 underwent a clockwise rotation upon opening (counterclockwise for closure), it seemed possible that the crystal structure may not reflect the fully closed MscL channel. The authors noted this possibility in their original publication (Chang et al., 1998). We speculated that G26, rather than V23 truly formed the constriction site, and that a slight counterclockwise rotation of TM1 was necessary for full closure (consistent with the rotation predicted from EPR studies). Although the evidence was not definitive, we have been, and plan on continuing to utilizing alternative approaches to test this hypothesis.

C1iii) Utilizing the Substituted Cysteine Accessibility Method (SCAM) to identify residues exposed in the closed and opening states of the MscL channel

One approach that has been often utilized for identifying residues within a channel pore is the substituted cysteine accessibility method (SCAM) (Akabas and Karlin, 1999). In the SCAM, cysteine substitutions are generated within the protein and sulfhydryl reagents are allowed to react upon gating. Hydrophilic, often charged, reagents are typically used to assure that only residues that are accessible to the aqueous environment are modified. If the residue is buried within the closed channel but exposed upon gating, then the reagent will react with the cysteine only upon channel opening. For channels with small pore sizes, a decrease in conductance is often observed upon this modification.

Batiza *et al.*, studying *E. coli* MscL, adapted the SCAM to test for accessibility *in vivo* (Batiza et al., 2002). This group exploited previous observations demonstrating that hydrophilic or charged substitutions within TM1 often lead to decreased viability (Ou et al., 1998; Yoshimura et al., 1999). This appears to be due to channel mis-gating, as patch clamp analysis demonstrated that these channels gate at lower than normal membrane tensions. In addition, reaction of charged sulfhydryl reagents with a cysteine mutant, G22C, demonstrated that these changes could be effected *in situ*, as assayed by patch clamp (Yoshimura et al., 2001). Batiza *et al.* developed an *in vivo* assay to test the accessibility of a mutant with a single substitution, L19C, and found a decreased viability upon treatment of the charged sulfhydryl reagent, [2-(Trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET). This decrease in viability was observed only when the channel was gated by an acute osmotic downshock, suggesting that the residue was only exposed upon gating.

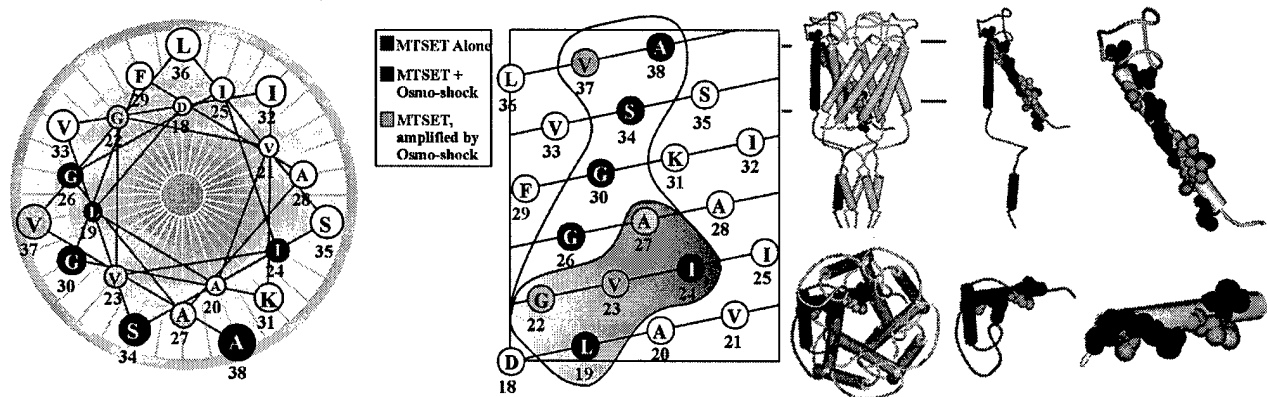


Fig. 7) Relative position of reactive TM1 Residues. An idealized helical wheel and helical net (left) and the structure of the closed or nearly-closed *M. tuberculosis* MscL channel derived from X-ray crystallography (top right) and a model for the closed structure of the *E. coli* channel (bottom right), which was derived from it, are shown. Residues have been colored according to the condition under which decreased viability was seen: blue indicates that the residues respond to MTSET alone; pink residues respond to MTSET alone but show an increased response upon osmotic downshock; residues in red require both MTSET and osmotic downshock to show decreased viability. Within the helical net, enclosed residues react with MTSET; those within the shaded region are residues in the gate that show increased accessibility to MTSET when the channel is gated by osmotic downshock. For the structural models on the right, the approximate boundary of the membrane is indicated with green horizontal lines. Residues in TM1 that react with MTSET are shown in CPK format. Note that the blue-labeled residues are not totally facing the lumen; a slight counter-clockwise rotation of TM1 would be required, suggesting modifications to models of the closed structure. In addition, for I24 to be exposed upon gating, a large clockwise rotation of TM1 would be required. (From (Bartlett et al., 2004))

We utilized the cysteine-scan mutant library discussed above to extend this finding. We tested every mutant in this library with this modified *in vivo* SCAM using MTSET (Bartlett et al., 2004) (**included in the appendix**). Testing for access in the presence or absence of osmotic downshock allowed us to resolve aspects of the structure of MscL in different conformational states, while it still resides in a living cell (**Fig. 7**). Our results were consistent with the overall predictions of the crystal structure; however, they suggest modifications needed to define the fully-closed state of the channel: we predict that a counterclockwise rotation of TM1 is required for full closure of the channel. In addition, residues exposed upon osmotic downshock, including I24C, suggest that a large clockwise rotation of this domain occurs upon channel gating. These findings support and extend predictions generated by EPR spectroscopy (Perozo et al., 2002), but

are not consistent with the counter-model for gating that proposes a slight rotation in the opposite direction (Sukharev et al., 2001; Betanzos et al., 2002). Note that further investigation is required to access how MTSET and other sulfhydryl reagents influence the channel activity.

C2) Engineering metal binding sites in the sensor pore

The experiments presented above predicted the aqueous accessibility of several residues in the closed state and upon channel mechano-activation and gating. We therefore wanted to determine if it was possible to design metal binding sites within the pore.

Because the cysteine library existed and metals have been shown to bind to multiple cysteine residues, our first attempt in looking for a 'proof-of-principle' for metal binding was utilizing one of the cysteine mutants. The R13 residue within *E. coli* MscL channel has been found to have several interesting properties. R13C confers a GOF phenotype, spontaneously forms disulfide bridges in excised patches, and when exposed to the sulfhydryl reagent MTSET, the GOF phenotype is partially remediated (Bartlett et al., 2004; Levin and Blount, 2004). These data imply an interesting correlation between the charged state of this residue and the dynamics of this region during gating, suggesting it as a prime candidate for testing the mutated MscL channel for metal-induced functional changes. Preliminary experiments with R13C demonstrated that an 80% increase in stimulus was necessary to gate the channel when 500 μM Cd^{++} was added to the bath of an excised patch (not shown). However, because R13C spontaneously forms disulfide bridges, channel activity could only be observed routinely in the presence of DTT, thus complicating the interpretation. We therefore generated the R13H mutant, which retains the ability to bind metals but will not form intersubunit disulfide bridges.

The function of R13H was found to be pH-dependent *in vivo*. As seen in **Fig. 8**, *E. coli* cells expressing this mutant grew similar to those expressing wild type MscL at lower pH; however,

attenuated growth was observed at higher pH. The channel activity was characterized in giant spheroplasts by patch clamp. The channels had “flickery” kinetics (shorter open dwell time) at all pH’s assayed (not shown). Shorter dwell times is one of the properties previously reported for MscL channels with mutations near the pore (Ou et al., 1998; Yoshimura et al., 1999; Maurer and Dougherty, 2003; Levin and Blount, 2004). Consistent with the *in vivo* observations, patch clamp results confirmed that the channel was more sensitive to mechanical stimuli at higher pH, while no difference was noted in the pressure required to open the wild type versus the R13H mutant at pH 6.0 (220 ± 30 versus 230 ± 30 mm Hg; $p = 0.7$; $n \geq 6$). Shifting the bath to pH 9.0 led to a decrease in the threshold pressure for both the wild-type and the R13H mutant MscL. However, this decrease in threshold was more profound for R13H than for wild type MscL ($28 \pm 3\%$ versus $15 \pm 3\%$, $p < 0.02$). These pH effects were reversible (not shown). These data suggest that removing the charge at the R13 position leads to a channel that mis-gates *in vivo*, thus conferring a GOF phenotype; reconstitution of this charge increases the threshold for the channel, and suppresses the phenotype. Hence, we noted that this channel could be used in instances in which we may want a sensor that could be easily modulated; this could have application for a sensory device.

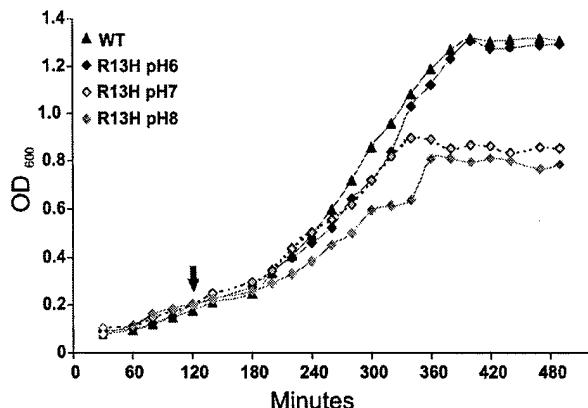


Fig. 8. The R13H MscL sensor mutant is modulated by pH. A growth curve of cells expressing the mutant sensor is shown. This sensor was later shown in electrophysiological characterization to have altered mechanosensitivity at different pH's. This is an example of how microbial physiology can indicate sensors with interesting properties that may be beneficial in a mechanosensor.

Consistent with the preliminary results with R13C (above), R13H mechanosensitivity was affected by the presence of Ni^{++} or Cd^{++} ions (Fig. 9). An increase in pressure threshold was observed. This influence on channel activity was observed at concentrations in the tens to

hundreds of μM and achieved saturation at about 2 mM (**Fig. 10A**). This effect was reversible upon perfusion with a solution free of the metal ions (**Fig. 10B**). Neither metal affected pressure threshold of the wild type channel. These data suggest a direct interaction of the metals with the substituted histidines exposed to the channel lumen, as opposed to an influence of these divalent cations on lipid fluidity or other indirect effects.

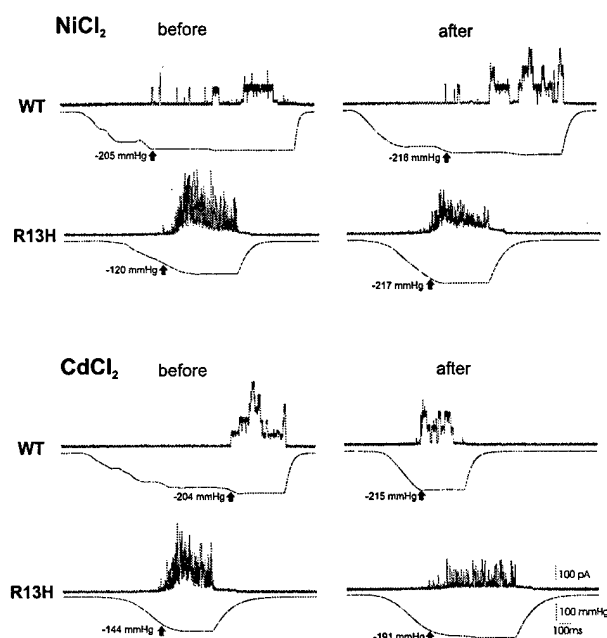


Fig. 9. The R13H MscL mutant bound metal ions, as indicated by changes in mechanosensor activity. Note that both Ni^{++} and Cd^{++} ions inhibited the R13H, but not the wild type sensor from activating. This strongly suggests that the metal ions bound the metals in a closed or near-closed state of the channel.

The metal-dependent increase in channel threshold was also found to be strongly influenced by pH. Metals bind histidine-containing proteins preferentially at higher pH where these residues are electrically neutral (Cherny and DeCoursey, 1999; Paddock et al., 2003). For R13H, the increase in pressure threshold observed in the presence of Cd^{++} was six times greater at pH 8.0 than at 6.0. Similarly, a three-fold increase was observed for Ni^{++} (**Figure 10C**) ($p < 0.001$ by unpaired Student's *t*-test for both Ni^{++} and Cd^{++}). That this change occurs near the expected pKa value for a histidine residue within a protein further supports the hypothesis that the metal ions are directly interacting with the histidine residues.

These controls are all consistent with the generation of a metal binding site within the MscL mechanosensor.

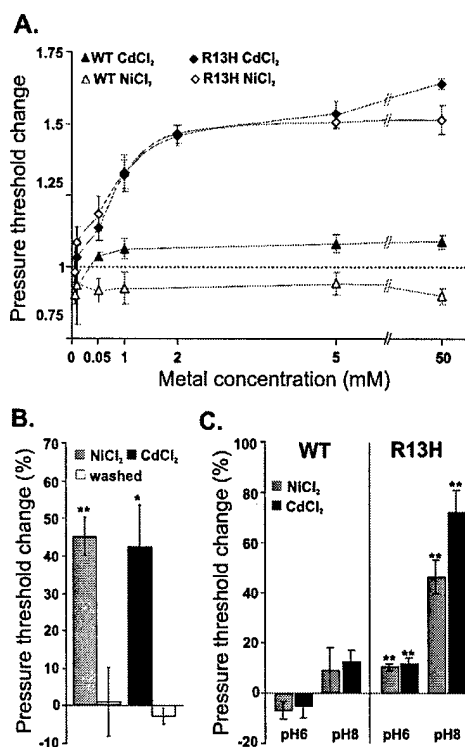


Fig. 10. All controls indicate that we have generated a metal binding site within the R13H mutant MscL mechanosensor. The influences on sensor activation was found to be concentration dependent (A), reversible by washing out the metal (B), and pH dependent in the region of the pK_i of a histidine within a protein molecule (C).

As described above, we had evidence that several residues, including, L19, G22, V23, I24 and G26, faced the channel lumen (Bartlett et al., 2004). To determine their exposure upon gating, we sequentially substituted each residue with histidine and assayed for metal-induced changes in activity by patch clamp. In *in vivo* experiments, each of these histidine mutants was found to confer a slowed-growth GOF phenotype. Further, patch clamp analysis demonstrated that these channels indeed have a lower gating threshold than wild type MscL. When expressed in PB104, a strain that expresses MscS, the ratio of the pressure threshold of MscL to that of MscS ranged from 0.72 to 1.22 for all of the above mutated channels, significantly lower than the wild type value of 1.48 ($p \leq 0.01$ for all mutants when compared to wild type using Student's *t*-test). These wild type ratios are consistent with values published previously (Blount et al., 1996c; Ou et al., 1998; Blount et al., 1999). In contrast to R13H, none of these histidine mutants showed any measurable pH modulation *in vivo* or *in vitro* (data not shown). Previous studies have demonstrated that the kinetics of wild type MscL can be well fit to three open dwell times, of which two are greater than 1 millisecond (Ou et al., 1998; Yoshimura et al., 1999; Levin and

Blount, 2004). In contrast, all of the calculated open dwell times for the above mutants were consistently less than 1 millisecond. The GOF phenotypes, decreased membrane tension thresholds and shorter dwell times are channel properties previously reported for MscL channels with mutations within the pore (Ou et al., 1998; Yoshimura et al., 1999; Maurer and Dougherty, 2003; Levin and Blount, 2004).

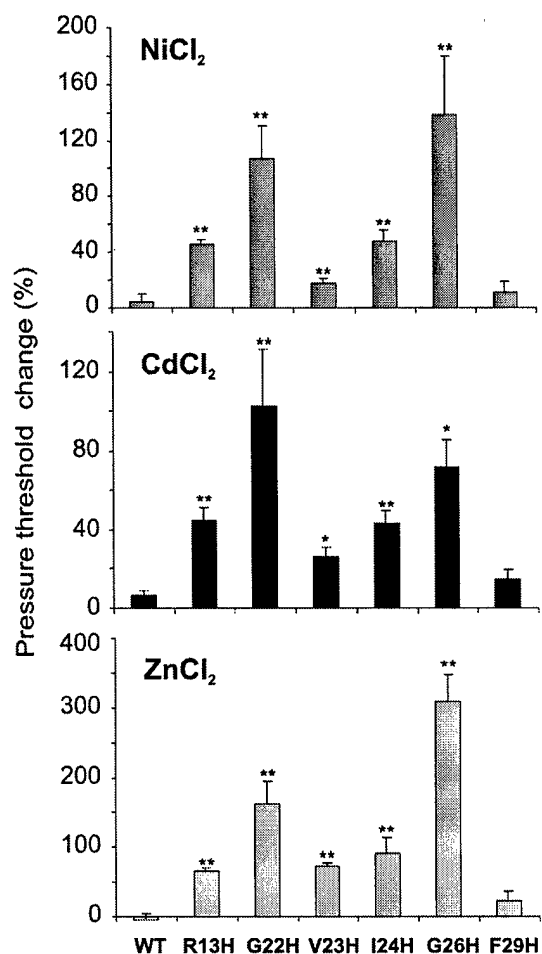


Fig. 11. Characterization of metal binding to histidine-mutated MscL sensors We compared Ni⁺⁺ (top), Cd⁺⁺ (middle) and Zn⁺⁺ (bottom) binding to mutants. We utilized data from other studies, including the *in vivo* SCAM study (Fig. 7 and accompanying discussion), to predict residues in the pore that, when mutated, would generate metal binding sites. Note that we now have several MscL sensor mutants that appear to contain metal binding sites.

The G22H, V23H, I24H and G26H mutants showed an increase in pressure threshold upon addition of Cd⁺⁺ or Ni⁺⁺ in the bath (**Figure 11, top and middle**). Similar to R13H, this effect was reversible and was observed at pH 8.0, but not at pH 6.0 (not shown). Together, these data strongly suggest that the metals bind to electrically neutral histidines within the lumen of the channel and increase the energy required for gating. Because zinc is well characterized and binds to histidine with a higher affinity than Ni⁺⁺ or Cd⁺⁺ (Krizek et al., 1993), we also tested all

mutants with this metal. As seen in **Fig. 11 (bottom)**, Zn^{++} not only gave results consistent with Cd^{++} and Ni^{++} , but yielded a larger effect (note the difference in scale of the Y-axis).

We also generated a histidine substitution at F29, a residue not predicted to be in the pore by Bartlett *et al* (2004). Although this mutant did confer a slowed growth GOF phenotype *in vivo*, it did not show a statistically significant decrease in membrane tension threshold (MscL/MscS pressure ratio of 1.30 ± 0.01 for F29, $n=19$; for wild type 1.48 ± 0.04 , $n=11$; $p = 0.056$); furthermore, in contrast to the histidine substitutions of residues predicted to line the pore an increase, rather than decrease, in the open dwell time was observed (τ_2 and τ_3 25 and 386 versus 6 and 33 msec for wild type; τ_1 is consistently < 1.0 msec (Li et al., 2004)). Also in contrast to mutations at positions predicted to project into the pore, no significant changes in F29H channel properties in the presence of metals were observed (**Fig. 11**).

D) Summary and future directions

The metal binding data are reviewed in **Fig. 12**. As shown, the data are consistent with a model of gating from previous data. We have demonstrated that we can indeed generate metal binding sites within the open or opening pore of the sensor. However, the metal binding does not appear to allow a significant electrical current, as determined by electrophysiological studies. This is likely due to the large size of the pore when the sensor is activated. However, with the models we have generated, we think we can now predict residues that will only be exposed in the activated state (e.g. I24).

Future studies will be directed at attempting to bind larger metal-based nanoparticles in the activated channel. This would have the advantage that many of these have semiconductor properties, which may have its own advantages. Tethering with sulfhydryl reagents to the entrance of the pore may significantly increase the efficiency of nanoparticle binding.

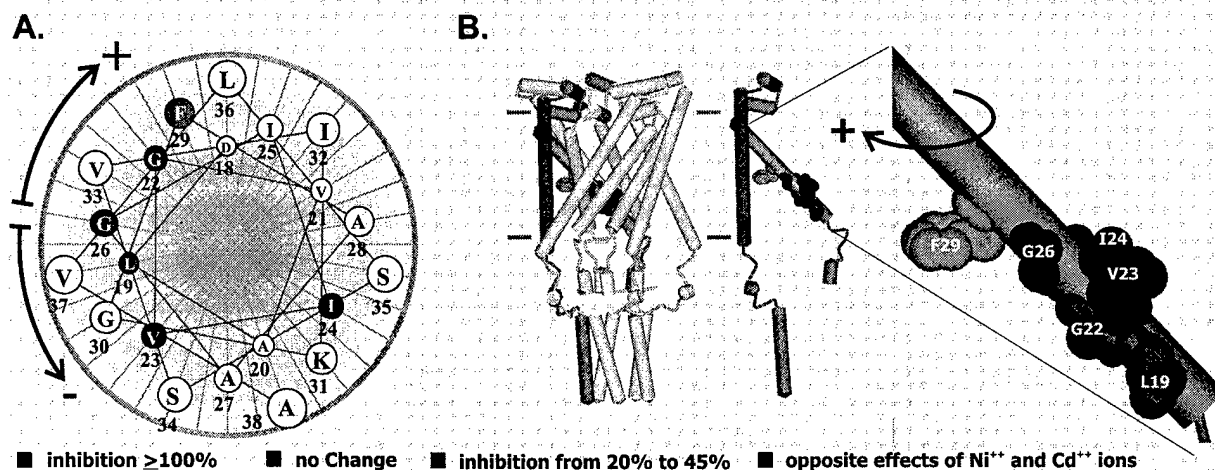


Fig 12) The location of the substituted residues, relative to a proposed constriction point, correlates with the degree of inhibition of channel gating by metal ions. (A) An ideal helical model of the relevant region of TM1 is shown. Residues colored in blue, when substituted with histidine, show the greatest inhibition by metal ions. These are proposed to be the constriction point (G26) and an additional residue (G22), which is clockwise to it (+ direction). Residues colored in red show inhibition to a lesser extent. L19H, in orange, shows opposite responses to Ni²⁺ vs. Cd²⁺. The green residue, F29H, is a control and showed no changes in channel properties in the presence of these metals. **(B)** A model of the *E. coli* MscL in a closed or nearly closed structure, derived from the *M. tuberculosis* crystallographic structure, is shown (left). A single subunit (middle) and an enlargement of the relevant region of TM1 (right) are shown. The arrow depicts the predicted clockwise rotation of TM1 upon channel gating. (from (Iscla et al., 2004))

In addition, we now have many mutated sensors that may have advantages for a mechanosensing nanodevice. For example, we have sensors that can be modulated not only by pH (described above) but can also be modulated by sulfhydryl reagents, and mutants that have extended activation times. If these modulatory elements could be combined in the proper combination, it could have great advantage for the device.

The other aspect of generating a device is the reconstitution. As stated above, we have found that the channel can be purified and reconstituted into biological membranes. However, the requirements for activation have not been well worked out. By reconstituting into biological and synthetic membranes we hope to determine what structural features (thickness, lipid headgroups and lateral pressure profiles) are of importance. More directly relevant to the project, we are now collaborating with AFOSR-funded researchers that are experts in self-assembly matrices to determine if some of the more recent findings can be used to accommodate the MscL sensor. In addition, we are presently trying to initiate collaboration

with AFOSR-funded researchers that have been placing membrane proteins in lipid matrices within microchips. Reconstitution into a stable and flexible matrix is perhaps the most challenging aspect of the project. However, if successful, it would be a revolutionary accomplishment, and it then would seem likely that a nanosensory device is feasible.

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